IN THE SPECIFICATION

Please add the following paragraphs after line 20 on page 7 as separate paragraphs:

The modified hydantoinases that may be used in the present invention were produced, identified and isolated using random mutagenesis procedures of the type described in U.S. Pat. Nos. 5,316935 and 5,906,930. Random mutagenesis protocols, which are also known as directed evolution procedures, are also described in Kuchner, O., Arnold, F. H. (1997) Directed Evolution of Enzyme Catalysts, TIBTECH 15:523-530; Chen, K. and Arnold F. (1991). Enzyme engineering for nonaqueous solvents--random mutagenesis to enhance activity of subtilisin E in polar organic media, Bio/Technology 9:1073-1077; Chen, K. and Arnold, F. (1993) Tuning the activity of an enzyme for unusual environments: sequential random mutagenesis of subtilisin E for catalysis in dimethylformamide, Proc. Natl. Acad. Sci. USA 90:5618-5622; and You, L. and Arnold, F. H. (1996). Directed Evolution of Subtilisin E in Bacillus Subtilis to Enhance Total Activity in Aqueous Dimethylformamide, Protein Engineering, 9, 77-83.

The random mutagenesis procedure used to identify and isolate the modified hydantoinases followed the same basic procedures as identified above. First, a large number of random mutations in the wild type nucleotide sequence (SEQ. ID. NO. 7) were generated. This library of nucleotide sequences where then used to express a large number of mutated enzymes. The library of mutated hydantoinases was then screened to identify those mutants with enhanced enzymatic activity and changed enantioselectivity.

The step of screening the first library of expressed amino acid sequences to identify desirable variants could have been accomplished using any number of suitable screening techniques which measure desirable enzyme properties. The screening method actually used was a pH-indicator assay which will be described in more detail below.

In accordance with the present invention, four enzymes having enhanced hydantoinase properties were identified as the result of the first round of random mutagenesis of the DSM 9711 nucleotide sequence (SEQ. ID. NO. 7). The first round mutant enzymes are 1CG7, 11DH7, 1BF7 and 19AG11. The nucleotide sequences for these first round mutants are set forth in SEQ. ID. NOS. 9, 11, 13 and 15, respectively. The corresponding amino acid sequences are set forth in SEQ. ID. NOS. 10, 12, 14 and 16, respectively.

A second round of random mutagenesis was conducted in which the 11 DH7 nucleotide sequence was randomly mutated to form a second library of mutants. A single mutant (22CG2) was identified which expressed a modified hydantoinase that exhibited desirable enzymatic properties. The 22CG2 enzyme is the same as the 11DH7 enzyme except that the 22CG2 mutant has an amino acid substitution at position 180.

The 22CG2 mutant was subjected to saturation mutagenesis in order to introduce all 20 different amino acids into amino acid position 95. 400 clones were screened and a mutant enzyme with enhanced enzymatic activity and higher (L)-selectivity was identified as Q2H4. The Q2H4 mutant is the same as the 22CG2 mutant except that phenylalanine is substituted for isoleucine at position 95.

As a result of the isolation and identification of the above identified mutants, it was established that improved hydantoinases may be obtained by modifying the DSM 9771 enzyme by substituting amino acids at positions 95, 124, 154, 180, 251 and 295. The substitutions may be made at one or more of the positions of SEQ. ID NO: 8. Table 1 sets forth preferred amino acid substitutions.

TABLE 1

Amino Acid Position	Substitution	Abbreviation
95	Ile.fwdarw.Phe	I95F
95	Ile.fwdarw.Leu	I95L
154	Val.fwdarw.Ala	V154A
180	Val.fwdarw.Ala	V180A
251	Gln.fwdarw.Arg	Q251R
295	Val.fwdarw.Ala	V295A

Amino acid substitutions other than those set forth in Table 1 are possible provided that the resulting hydantoinase exhibits desirable enzymatic properties. For example, other suitable amino acid substitutions for isoleucine at position 95 include Gly, Ala, Val, Leu, Phe, Tyr and Trp. For positions 154, 180 and 295, suitable alternative amino acid substitutions for valine include Ala and Gly. Suitable alternative amino acid substitutions at position 251 for glutamine include Arg, Lys and Asn. The amino acid substitutions may be made by saturation mutagenesis followed by screening of clones. The substitutions may also be made by chemical manipulation of the DSM 9711 enzyme or by conventional synthesis of peptides having the desired amino acid substitutions at the desired locations. It should be noted that the above listed amino acid substitutions are intended to be exemplary of preferred alternative substitutions at the various substitution sites. Substitutions of other amino acids are possible provided that the enzymatic activity of the resulting protein is not destroyed. The usefulness of a particular amino acid substitution at positions 95, 154, 180, 251 and 295 can be determined by routine pH screening as described below.

The amino acid substitutions described above may be made at equivalent positions in other hydantoinases. "Other hydantoinases" refers to enzymes that catalyze the hydrolysis of any 5'-mono- or disubstituted hydantoin derivative to yield the derived N-carbamoyl-amino acid and might have between 20 and 100% amino acid sequence identity to the hydantoinase from Arthrobacter sp. DSM 9771 which can be determined by sequence alignment algorithm

such as BLAST (Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990) "Basic local alignment search tool." J. Mol. Biol. 215:403-410).

Amino acid positions are numbered in a linear order (starting at the start codon) and not according to their functional and structural context. Therefore amino acid residues that contribute in the same way to an enzyme function of different hydantoinases do not necessarily have the same amino acid position number due to deletion or insertion events in the homologous enzyme. "Equivalent positions" of hydantoinases therefore refers to amino acid positions that contribute in the same way to a function (activity or enantio-selectivity) as the amino acids identified in our evolution experiment. If the amino acid sequence identity of different hydantoinases is high, for example higher than 60%, and the amino acid position is located in a conserved region without sequence gaps, equivalent positions can be determined by sequence alignment using for example the BLAST algorithm. If the amino acid sequence identity is low, for example lower than 60%, and the amino acid position is located in a non-conserved region, or near gaps without being surrounded by regions of conserved amino acids, other methods such as structure alignments can be used if x-ray structures are available (Mizuguchi, K., Go, N., Seeking significance in 3-dimensional protein-structure comparisons. Cur. Opin. Struc. Biol. 5:377-382 (1995)). Here, backbone atoms are structurally aligned and equivalent positions can be found based on the relative locations of the amino acid residues of the structures.

An amino acid position that is identified for example by directed evolution to contribute to a specific function can often be occupied by different amino acid residues, not just the one that was identified by random point mutagenesis. Some substitutions will destroy the function, some of them will not change the function, and yet others will improve the function. With known methods, such as site saturation mutagenesis, one can easily identify amino acids that contribute in the same way to a function or even improve it by

replacing the found amino acid residue with all possible amino acid residues (Miyazaki, K., Arnold, F., Exploring nonnatural evolutionary pathways by saturation mutagenesis: rapid improvement of protein function. J. Mol. Evol. 49:1716-1720). Even non-natural amino acids can be introduced at the identified site using a stop codon and a suppresser tRNA linked to a non-natural amino acid (Bain, J. D., Glabe, C. G., Dix, T. A., Chamberlain, A. R., Diala, E. S., Biosynthetic Site-Specific Incorporation Of A Non-Natural Amino-Acid Into A Polypeptide. JACS 111:8013-8014 (1989)).

Six modified hydantoinases in accordance with the present invention are listed in Table 2. Table 2 also lists the amino acid substitutions with respect to the DSM 9771 sequence (SEQ. ID. NO. 8) for each modified enzyme which is identified.

TABLE 2

Hydantoinase Variant	Amino Acid Substitution
1CG7 (SEQ. ID. NO. 10)	V154A
11DH7 (SEQ. ID. NO. 12)	I95L + O251R
1BF7 (SEQ. ID. NO. 14)	V295A
19AG11 (SEQ. ID. NO. 16)	I95L
22CG2 (SEQ. ID. NO. 18)	I95L + V180A + Q251R
Q2H4 (SEQ. ID. NO. 20)	I95F + V180A + Q251R

The modified hydantoinases of the present invention may be used in the same manner as other hydantoinases to produce optically pure D- and L-amino acids. For example, see Biocatalytic Production of Amino Acids and Derivatives (Rozzell, J. D. and Wagner, F. eds.) (1992) Hanser Publisher, NY, at pages 75-176, for a description of the use of hydantoinases in the production of optically pure amino acids from DL-5-monosubstituted hydantoins. The general use of hydantoinases is also described in Enzyme catalysis in organic synthesis (Dranz, K. and Waldmann, H. eds.) 1995, VCH-Verlag, Weinheim, at pages 409-431; and Wagner, T. et al. (1996) Production of 1-methionine from d,1-5-(2-methylthioethyl)

hydantoin by resting cells of a new mutant strain of Arthrobacter species DSM 7330, Journal of Biotechnology 46:63-68.

Amino acids referred to in the present invention are all natural or unnatural amino acids, wherein the amino acids are deemed to be a primary amine connected to carboxylic acid group via one intermediate C-atom (α-C-atom). This C-atom bears only one further residue. Preferred unnatural amino acids are disclosed in DE 19903268.8. Preferred natural amino acids are those mentioned in Beyer-Walter, Lehrbuch der Organischen Chemie, 22. Auflage, S. Hirzel Verlag Stuttgart, S.822-827. Among those amino acids presented above alanine, leucine, isoleucine, methionine, valine, tert-leucine or neopentyl glycine are not preferably transformed in a process utilizing the modified hydantoinase.

To transform hydantoins directly to the amino acids by enzymes it is preferred to use a whole-cell catalyst which includes the hydantoinase of the invention accompanied with a carbamoylase. A hydantoin racemase can also be used in addition to the hydantoinase and carbamoylase.

The hydantoinase can be used within this process either in their free or immobilized form. Also the carbamoylase and hydantoin racemase may be immobilized, too. Techniques to immobilize enzymes are well known to the skilled worker. Preferred methods are mentioned in Bhavender P. Sharma, Lorraine F. Bailey and Ralph A. Messing, Immobilisierte Biomaterialien-Techniken und Anwendungen, Angew. Chem. 1992, 94, 836-852; Dordick et al., J. Am. Chem. Soc. 194, 116, 5009-5010; Okahata et al., Tetrahedron Lett. 1997, 38, 1971-1974; Adlercreutz et al., Biocatalysis 1992, 6, 291-305; Goto et al., Biotechnol. Prog. 1994, 10, 263-268; Kamiya et al., Biotechnol. Prog. 1995, 11, 270-275; Okahata et al., Tibtech, February 1997, 15, 50-54; Fishman et al., Biotechnol. Lett. 1998, 20, 535-538).

The transformation discussed can be conducted in a batch process or continuous manner. Advantageously, an enzyme-membrane-reactor is used as the reaction vessel (Wandrey et al. in Jahrbuch 1998, Verfahrenstechnik und Chemieingenieurwesen, VDI S. 151ff.; Wandrey et al. in Applied Homogeneous Catalysis with Organometallic Compounds, Vol. 2, VCH 1996, S.832 ff.; Kragl et al., Angew. Chem. 1996, 6, 684f.).

A further embodiment of the present invention is directed to a whole cell catalyst comprising a gene encoding for a carbamoylase, an optional racemase and a hydantoinase wherein the hydantoinase is considered to be according to the modified hydantoinase of the invention.

Advantageously, a bacteria is used as a cell, because of high reproduction rates and easy growing conditions to be applied. There are several bacteria known to the skilled worker which can be utilized in this respect. Preferably *E. coli* can be used as the cell and expression system in this regard (Yanisch-Perron et al., Gene (1985), 33, 103-109). Another aspect of the invention is a process for the production of enantiomerically enriched amino acids, which utilizes a whole cell catalyst according to the invention.

It is further preferred in this respect that amino acids like methionine, threonine, lysine or tert-leucine are produced by the aid of the whole cell catalyst.

The transformation discussed in this instance can be conducted in a batch process or continuous manner. Advantageously, an enzyme-membrane-reactor is used as the reaction vessel (Wandrey et al. in Jahrbuch 1998, Verfahrenstechnik und Chemieingenieurwesen, VDI S. 151ff.; Wandrey et al. in Applied Homogeneous Catalysis with Organometallic Compounds, Vol. 2, VCH 1996, S832 ff.; Kragl et al., Angew. Chem. 1996, 6, 684f; DE 19910691.6).

There is a further aspect of the invention, which is directed to a process for the production of a whole cell catalyst of the invention. The process is preferably conducted by

using expression vectors pOM17, pOM18, pOM20, pOM22 and/or pOM21. In addition primers of SEQ. NO. 23, SEQ. NO. 24, SEQ. NO. 21 and/or SEQ. NO. 22 are used with regard to the production of the whole cell catalyst.

Examples of Practice are as follows:

EXAMPLE 1

The following example provides additional details regarding the procedures used to identify and isolate the modified hydantoinases in accordance with the present invention.

The hydantoinase from Arthrobacter sp. DSM 9771 (U.S. Pat. No. 5,714,355) was cloned by polymerase chain reaction (PCR). The nucleotide sequence was determined and compared to other hydantoinases from closely related Arthrobacter strains. The nucleotide and amino acid sequences for the hydantoinase are set forth in SEQ. ID. NOS. 7 and 8, respectively. The cloned enzymes from Arthrobacter sp. DSM 9771 share about 97.5% identity based on their nucleotide sequence (corresponding to 7 amino acid changes) with the enzymes from Arthrobacter aurescens DSM 3747 and DSM 3745. The enzymes were expressed in *E. coli* JM109 using a rhamnose inducible vector construct which was provided by the Institute of Industrial Genetics, Universitait Stuttgart (Germany).

The hydantoinase was subjected to random mutagenesis using error-prone PCR. Approximately 10,000 clones were screened using a pH-indicator assay as described below:

1. Seed culture plates: plates containing 100 µl/well LBamp were inoculated with single colonies/well and incubated for 24 hours at 30° C., 250 rpm.

2. Main culture plates: cells from seed culture plates were transferred with a 96-pin replicator into plates containing 200 µl LBamp+0.2% rhamnose. Plates were incubated for 24 hours at 30°C, 250 rpm.

3. Assay: using a pipetting robot, the culture of each well was mixed by pipetting up and down (3×) and transferred (75 µl each) into two fresh plates. The two plates are filled with 100 µl/well freshly prepared substrate solution (80 mM D-MTEH and L-MTEH respectively, in 0.05 g/l cresol red pH 8.6). The absorbance at 580 nm is measured immediately after the substrate was added to the plate and after 3 hours incubation at room temperature. The activity was calculated as follows:

Activity=(A580(0h)-A580(3h))/((A580(0h)-0.8))(Rem:0.8 is the absorbance without cells)

For screening purposes, the ratio of activities for the D- and L-enantiomers is taken as an indicator for changed enantioselectivity.

Since the ratio of activities for different enantiomers in the screening tests is only a first hint of enantioselectivity, the identified mutants were confirmed by chiral HPLC using the racemic substrate as follows. 2 ml overnight cultures were added to 2 ml 80 mM DL-MTEH in 0.1M Tris pH8.5 and incubated at 37°C. After 1h and 2h respectively, the reaction mixture was centrifuged for 2 minutes, 14000 rpm. 20 µl of the supernatant was applied onto the HPLC column and the various fractions eluted.

About 2% of the population showed a significantly higher (>50%) activity compared to wild-type DSM 9771. Although a considerable number of those clones might be false positives due to common variation of expression level in a population, about 50% of rescreened clones were indeed higher activity mutants. The high number indicates that hydantoinase has a large evolutionary potential (its activity and enantioselectivity can be therefor improved). This can be rationalized since a high Km value (about 15 mM), a rather low specific activity (about 12U/mg) and a low expression level (<10% of total protein) leaves room for improvements of this enzyme.

Table 3 shows the results of the tested mutants. Mutant 1CG7 shows a dramatic increase of (D-) selectivity. Compared to wild-type, the enantiomeric excess of the product is 4 times increased. The enantioselectivity of clone 11DH7 and 19AG11 was changed into the opposite direction since both mutants are absolutely non-selective. The activity mutant 1BF7 possesses the same enantioselectivity as wild-type.

TABLE 3

Clone	Conversion	enantiomeric excess [%]
wild-type	42% after 2 hours	19
1CG7	42% after 2 hours	90
11DH7	42% after 2 hours	0
19AG11	37% after 2 hours	0
1BF7	45% after 1 hour	19

All of the mutants were sequenced and the nucleotide and amino acid sequences established as set forth in Table 4.

TABLE 4

	Nucleotide Sequence (SEQ. ID. NO.)	Amino Acid Sequence (SEQ. ID. NO.)
1CG7	9	10
11DH7	11	12
1BF7	13	14
19AG11	17	18

A second round of random mutagenesis was conducted using the first generation mutant 11 DH7 as the parent.

Two different libraries with different error rates (20% and 50% inactive clones) were produced and 10,000 clones of each library were screened using the above-described pH-indicator method. None of the screened clones showed significantly higher L-selectivity but mutants with improved activity and higher D-selectivity were found. One mutant (22CG2) differing in only one amino acid (V180A) from the parent was found to be 4-fold more active compared to parent 11DH7.

Sequencing of the first generation mutants 11DH7 and 19AG11 revealed a single mutation (I95L) is responsible for their decreased D-selectivity. Introducing all 20 different amino acids into amino acid position 95 of mutant 22CG2 by saturation mutagenesis and screening of about 400 clones revealed a new mutant (Q2H4) with significantly improved L-selectivity (ee_L =20%) and 1.5-fold improved activity compared to its parent 22CG2. The nucleotide and amino acid sequences for 22CG2 are set forth in SEQ. ID. NOS. 17 and 18, respectively. The nucleotide and amino acid sequences for Q2H4 are set forth in SEQ. ID. NOS. 19 and 20, respectively.

In addition to the improvements provided by the mutants described above, the activity of the whole cell catalyst could be increased by a factor of 10 by addition of 1 mM manganese to the growth medium and to the substrate solution. Under those conditions the activity of mutant 22CG2 was determined to be about 380 U/gCDW which is a 50-fold increase in activity compared to the activity described for Arthrobacter sp. DSM 9771.

All of the modified enzymes identified in accordance with the present invention have activities and/or enantioselectivity which are better than the unmodified DSM 9771 hydantoinase. When tested under standard conditions by HPLC, the Q2H4 mutant showed inverted enantioselectivity for the hydrolysis of D,L-MTEH. Q2H4 produced N-carbamoyl-L-methionine with an enantiomeric excess (ee) of 20% at about 30% conversion. In addition, the Q2H4 mutant was approximately 1.5-fold more active than its parent 22CG2.

EXAMPLE 2

In a further example, L-methionine was produced with a recombinant whole cell catalyst. Recombinant whole cell catalysts were prepared by co-expressing the evolved or wild-type hydantoinase with a hydantoin racemase and a L-carbamoylase in *E. coli* as

follows.

Strains and expression vectors. The L-carbamoylase and hydantoinase expression vector pOM17 and pOM18 were constructed by PCR amplification of the hyuC and hyuH gene, respectively, from Arthrobacter sp. DSM 9771 using the following primer: for hyuC-amplification: 5'-AGGCGACATA-TGACCCTGCAGAAAGCGCAA-3' (SEQ. ID. NO. 23), 5'-ATGGGATCCCCGGT-CAAGTGCCTTCATTAC-3' (SEQ. ID. NO. 24); for hyuH-amplification: 5'-AGAACATATGTTTGACGTAATAGTTAAGAA-3' (SEQ. ID. NO. 21), 5'-AAAAGGAT-CCTCACTTCGACGCCTCGTA-3' (SEQ. ID. NO. 22). The amplified fragments were cleaved with the restriction enzymes Ndel and BamHI and inserted using the same restriction enzymes downstream the rha BAD promoter (rhamnose promoter) into the vector pJOE2702 (Volff, J.-N., Eichenseer, C., Viell, P., Piendl, W. & Altenbuchner, J. (1996) Nucleotide sequence and role in DNA amplification of the direct repeats composing the amplificable element AUDI of Streptomyces lividans 66. Mol. Microbiol. 21, 1037-1047). The co-expression plasmid pOM20 comprising the L-carbamoylase and hydantoinase gene, both separately under the control of a rhamnose promoter, was derived from Plasmid pOM17 and pOM18. pOM17 was digested by SaII and treated with the Klenow fragment to form blunt ends. pOM18 was digested by BamHI and also treated with the Klenow fragment to form blunt ends. Both fragments were subsequently digested from HindIu. The 1521 kb-fragment comprising the carbamoylase gene and rhamnose promoter derived from pOM17 was ligated with the 5650 kb-fragment of the digested pOM18 to yield pOM20. Mutations of the L-selective hydantoinase were introduced into pOM20 using the restriction enzymes RsrII and KasI which yielded pOM22. The racemase expression vector pOM21 was derived from pACYC184 (Rose, R.E. The nucleotide sequence of pACYC184. Nucleic Acids Res. 16, 355 (1988)) and carries a chloramphenicol selection marker and the racemase gene hyuR from Arthrobacter sp. DSM3747 under the control of the rhamnose promoter. All plasmids were

routinely transformed into *E. coli* JM109 (Yanisch-Perron, C., Viera, J. & Messing, J. (1984) Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13 mpl8 and pUC vectors. Gene 33, 103-109). The hydantoin converting pathway was installed in *E. coli* JM109 by transformation of pOM20 and pOM22, respectively into *E. coli* JM109 (pOM21). Cells were either grown in LB liquid medium or on LB-agar plates (Luria. S. E., Adams, J. N. & Ting, R. C. (1960) Transduction of lactose-utilizing ability among strains of Escherichia coli and Shigella dysenteriae and properties of phage particles. Virology 12, 348-390), both supplemented with the respective antibiotics for the growth and expression medium (100 μg/ml ampicillin, 50 μg/ml chloramphenicol) and addition of 2 mg/ml rhamnose for the expression medium.

Error-prone PCR. Random mutagenesis of the hydantoinase gene was performed in a 100 μl reaction mix containing 0.25 ng of plasmid DNA as template, Boehringer PCR buffer (10 mM Tris, 1.5 mM $MgCl_2$, 50 mM KCI, pH 8.3), 200 μM dATP, 200 μM dTTP, 200 μM dGTP, 200 μM dCTP, 50 pmol of each primer, and 2.5U Taq polymerase (Boehringer). After 30 cycles, the 1667 amplification product was extracted from gel using the Qiaexll gel-extraction kit (Qiagen, Valencia, Calif.) and subdloned into vector pJOE2702 using the EcoRI and HindIII restriction sites. Religation frequency of alkaline phosphatase treated vector was below 1%.

Saturation mutagenesis. For randomization of the codon for amino acid position 95, the QuickChange.TM. protocol (Stratagene, La Jolla, Calif.) was used. About 10 ng plasmid from clone 22CG2 were amplified by PCR using two complimentary oligonucleotides (5'-CATCGAGATGCCGNNNACCTTCCCG-CCCAC-3', 5-GTGGGCGGGAAGGTNNNCGGCATCTCGATG-3'). After PCR amplification the

reaction mixture was treated for 2 hours with 20 U of the restriction enzyme DpnI. Transformation of 10 µl DpnI digested reaction mixture into competent cells yielded a library of more than 2000 mutants of which about 400 were screened.

Preparation of library and screening. Single colonies of transformed *E. coli* were transferred into 384-well plates (master plates) using the robot system Qbot (Genetix, Dorset, UK). After 20 hour growth at 37°C plates were stored at -80° C. For subsequent screening, plates were thawed and replicated into 96-well plates containing 200 .mu.l per well inductor medium. A Biomek 1000 pipetting workstation (Beckman, Fullerton, Calif.) was used to divide the 24 hours at 30° C incubated plate into two fresh 96-well plates one containing 100 µl 80 mM L-MTEH the other 100 µl 80 mM D-MTEH in 50 mg/l cresol red solution adjusted to pH 8.5. Initial absorbance at 580 nm and after 3 hours incubation at room temperature was measured using a THERMOmax plate reader (Molecular Devices, Sunnyvale, Calif.). Activity was calculated from the difference of initial and absorbance after 3 hours incubation divided by the cell density of each well. For the saturation mutagenesis library incubation time was reduced to 1.5 hours. The ratio of activity towards the L- and D-enantiomer was taken as a first indicator for enantioselectivity. Identified clones were then tested using the racemic substrate under conditions described below.

Characterization of activity and enantioselectivity. Plasmid of mutant found to be positive in the screen was sequenced and retransformed into *E. coli*. A culture of retransformed *E. coli* was grown for 16-18 hours (until OD10) in inductor medium supplemented with 1 mM $MnCl_2$. 2 ml substrate solution consisting of 80 mM D,L-MTEH, 0.1M Tris pH 8.5, 1 mM $MnCl_2$ (pre-incubated at 37°C.) were added to 2 ml cell culture (OD600-7). The reaction mixture was immediately incubated at 37°C. in a water bath. After different time periods (as

specified in the text) 1 ml samples were taken and centrifuged for 5 minutes at 14,000 rpm. 20 µl of supernatant were analyzed by chiral HPLC using a column manufactured by Degussa-Huels AG. Activity was calculated from the amount of produced N-carbamoyl-D,L-methionine and expressed as U/ml cell culture of U/mg cell dry weight (CDW) were 1U is the amount of whole-cell catalyst to produce 1 µmol N-carbamoyl-D,L-methionine in one minute under stated reaction conditions. Enantioselectivity of the hydantoinase and its mutants were compared by calculating the percentage of ee.sub.D ((D-L)/(D+L)) and ee_L ((L-D)/(L+D) respectively for the product at various extents of conversion. A conventional determination of E (enantiomeric ratio) from ee-values and the extent of conversion as described by Chen et al. (Chen, C. S., Fujimoto, Y., Girdaukas, G. & Sih, C. J. (1982) Quantitative analysis of biochemical kinetic resolutions of enantiomers. J Am. Chem. Soc. 104, 7294-7299) is not possible because of the fast racemization of the substrate.

Conversion of D,L-MTEH into L-met. 8 mg cell dry mass of *E. coli* JM109 (pOM20 & pOM21) and *E. coli* JM109 (pOM 22 & pOM21) were added to 4 ml 100 mM D,L-MTEH in 0.1 M Tris pH 7.8 supplemented with 1 mM $MnCl_2$. The reaction mixture was incubated at 37°C. Samples were analyzed after indicated periods of time and analyzed by HPLC for MTEH, D,L-C met, and D,L-met as described in Volkel, D. & Wagner, F. Reaction (1995) mechanism for the conversion of 5-monosubstituted hydantoins to enantiomerically pure L-amino acids. Ann. NY Acad. Sci. 750, 1-9. The optical purity of the compounds was analyzed by chiral HPLC as described above.

The conversion of D,L-MTEH into L-met is significantly improved for the catalyst with the evolved hydantoinase. After three hours, approximately 60 mM L-met was produced from 100 mM D,L-MTEH, whereas the whole cell catalyst with the wild-type pathway produced only 10 mM of the amino acid. The concentration of the D-C met

intermediate was reduced by a factor of 4 and the productivity for the production of L-amino acid was 8-fold increased during the first hour of the reaction.

EXAMPLE 3

The following example shows that production of L-methionine was significantly improved with an evolved hydantoinase of mutant 22CG2 which has improved activity and is not enantioselective (0% enantiomeric excess at 42% conversion, see Table 3). Mutations of the evolved hydantoinase from mutant 22CG2 were introduced into pOM20 as previously described using the restriction enzymes RsrII and KasI, which yielded pOM23. This co-expression vector was transformed into *E. coli* JM109 (pOM21). The resulting whole cell catalyst *E. coli* JM109 (pOM21/pOM23) and *E. coli* JM109 (pOM21/pOM20) were used for conversion of D,L-MTEH into methionine. 125 mg cell dry mass of the respective cells were added to 5 ml substrate solution (100 mM D,L-MTEH in 0.9% NaCl, 1 mM $MnCl_2$, pH 7.8) and incubated for 1 hour at 37°C. The whole cell catalyst with the improved hydantoinase from clone 22CG2 produced about 65 mM methionine within one hour whereas whole cell catalyst with the wild-type hydantoinase produced only 8 mM during the same reaction time. This demonstrates that an evolved hydantoinase without significant enantioselectivity but improved activity leads to a significant improvement for the production of methionine.

Page 11 (Abstract), after the last line, beginning on a new page, please insert the attached substitute Sequence Listing.